Ultraviolet Absorption Method for the Determination of Polyunsaturated Constituents in Fatty Materials*

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INTRODUCTION

VEGETABLE oils and animal fats are complex mixtures consisting essentially of saturated and unsaturated mixed fatty acid glycerides. The distribution of fatty acid components in different fatty materials varies considerably, and the complete analysis for these components in a given material poses a complex problem. The fatty acid constituents of unsaturation higher than oleic acid (1 double bond) likely to be present are linoleic (2 double bonds), linolenic (3 double bonds), and arachidonic acids (4 double bonds), and their conjugated isomers. Because of the high reactivity of these constituents, their presence even in minor and trace proportions may have considerable significance in research, industrial, and food uses of fats and oils.

The chemical method of analysis for the non-conjugated polyunsaturated constituents, linoleic, linolenic, and arachidonic acids, based on determination of iodine number and thiocyanogen number, is time-consuming and is inaccurate when these compounds are present in small proportions. Supplementary insoluble bromide tests are likewise insensitive and unreliable in such cases. No reliable chemical method exists for determining the minor conjugated constituents of oils and fats.

Recently proposed spectrophotometric methods offer the possibility of furnishing a method of analysis for these polyunsaturated constituents which is superior to chemical methods in speed, sensitivity, and specificity. Direct ultraviolet spectrophotometric analysis for the conjugated components is relatively simple, involving difficulties only when they are present in small proportions. Ultraviolet spectrophotometric analysis for the non-conjugated components, which have no absorption maxima above 200 mμ, may be accomplished by converting them to their conjugated isomers by alkali-isomerization followed by ultraviolet absorption measurements, as shown by Mitchell, Kraybill, and Zscheile,[1] and by Beadle and Kraybill.[2] This method also involves difficulties when only small proportions of these constituents are present.

The purpose of the present investigation was to combine, modify, and improve existing spectrophotometric methods for application principally to materials having small proportions of polyunsaturated constituents, such as animal fats and their soaps, partially hydrogenated fats, and purified fatty preparations.

The modified method proposed here comprises measurement of the ultraviolet absorption of a sample before and after isomerization, the latter in an improved medium; correction of data for extraneous absorption; correction of data for conjugated constituents originally present and remaining undestroyed by the isomerization treatment; and calculation of the proportions of conjugated and non-conjugated diene, triene, and tetraene fatty acid constituents in the sample.

Throughout this paper, specific extinction coefficient, k, will be defined as

$$k = D/bc = (-\log T)/bc,$$

where D is the spectral density and T the transmittance of a solution relative to that of the solvent in an equal cell, b is the inside length (in centimeters) of the cell used, and c is the concentration of the solution in grams per liter of solution. All data were obtained with a Beckman Model DU photoelectric spectrophotometer, using a hydrogen lamp and an absorption cell compartment for cells up to 10 cm long.

CONJUGATED CONSTITUENTS

Bradley and Richardson[3] have analyzed certain vegetable oils for conjugated fatty acids

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[1] J. H. Mitchell, Jr., H. R. Kraybill, and F. P. Zscheile, Ind. Eng. Chem. Anal. Ed. 15, 1 (1943).

[2] B. W. Beadle and H. R. Kraybill, J. Am. Chem. Soc. 66, 1232 (1944).

[3] T. F. Bradley and D. Richardson, Ind. Eng. Chem. 34, 237 (1942).

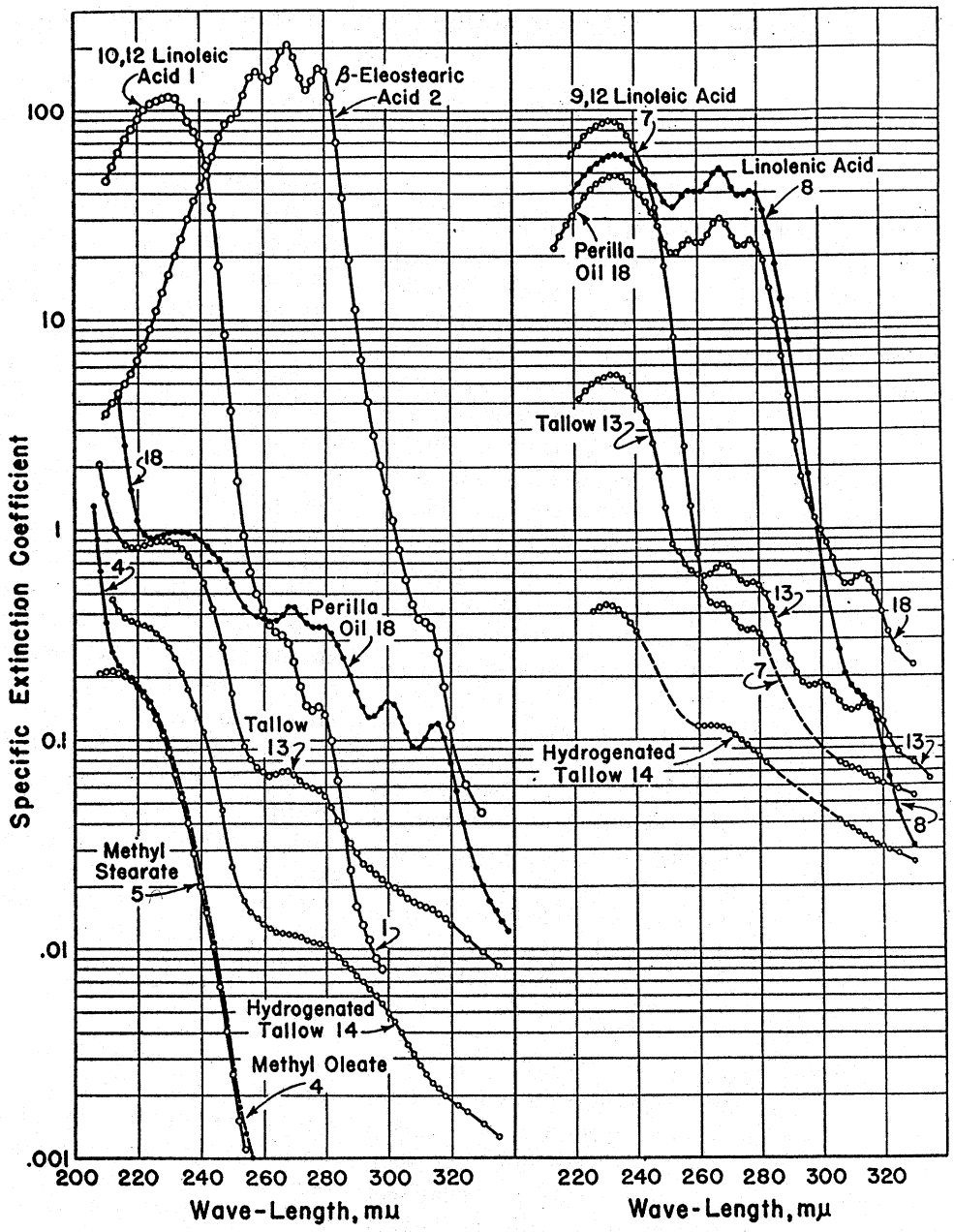

FIG. 1. Ultraviolet absorption curves for ethanol solutions of purified β-eleostearic acid and 10,12 linoleic acid standards; neohexane solutions of purified methyl oleate and methyl stearate, and typical oil and fat samples containing small proportions of conjugated fatty acid glycerides. Slit widths 1.5 to 2 mμ.

FIG. 2. Ultraviolet absorption curves for ethanol solutions of fatty materials after isomerization in KOH-glycerol solution for 30 minutes at 180°C: purified 9,12 linoleic acid and linolenic acid standards; and the typical oil and fat samples shown in Fig. 1. Slit widths approximately 3.5, 2.5 and 4.5 mμ at 232, 268, and 316 mμ, respectively.

TABLE I. Ultraviolet absorption data for purified fatty acids and esters: observed or reported values for wave-length λ_{max} and specific extinction coefficient k at absorption maximum used; values tentatively adopted as standard in analysis for conjugated fatty acid constituents; and ratio of k value at λ_{max} to average value of k at $\lambda_{max}+6$ mμ and $\lambda_{max}-6$ mμ, i.e., $k/\Delta k$.

Sample No.	Double bonds	Compound	Solvent	Observed			Used in analysis		
				mμ λ_{max}	k	$k/\Delta k$	mμ λ	k	$k/\Delta k$
1	2[a]	10,12 linoleic acid	Neohexane	231	119	—	232	119	—
			Ethanol	231	117	—			
2	3[a]	β-eleostearic acid	Neohexane	267	214	2.71	268	214	2.8
			Ethanol	268	209	2.78			
—	4[a]	Parinaric acid[b]	Ethanol	320	203	1.7	316	220	2.5
—	4[a]	Tetraene acid from isomerized arachidonic acid	Ethanol	315[c]	220	2.5[d]			
3	1	Methyl oleate	Ethanol	232[e]	.077	—			
4	1	Methyl oleate	Neohexane	232[e]	.072	—	232	.07	
4-A	1	Oleic acid	Neohexane	232[e]	.032	—	232	.03	
5	0	Methyl stearate	Neohexane	232[e]	.070	—	232	.07	
6	0	Stearic acid	Neohexane	232[e]	.025	—	232	.03	

[a] Conjugated.
[b] Data obtained by comparator measurements on Fig. 1 of reference 6.
[c] From reference 12.
[d] See footnote 13.
[e] No maxima or inflections observed at this wave-length.

simply by calculating the ratios of the specific extinction coefficients for the oils to those for purified 9,11 linoleic acid,[4] α-eleostearic acid,[5] and parinaric acid,[6] at the wave-lengths of the absorption maxima of the acids, assuming that all the observed absorption for the oils at these wave-lengths was due to the conjugated fatty constituents. Brode, Patterson, Brown, and Frankel[7] have attempted to take into account the overlapping of the absorption curves of the diene, triene, and tetraene conjugated acids, and have set up simultaneous equations for calculating their proportions in mixtures. Kass[8] has applied a similar procedure to the analysis of drying oils, using more recent absorption data for purified conjugated acids. This method is particularly applicable to materials having substantial proportions of conjugated fatty acids and relatively free of interfering absorption by extraneous compounds.

The methods mentioned above do not yield accurate analyses for materials such as animal fats, many vegetable oils, and many fatty preparations, which ordinarily contain, in decreasing order of abundance, diene, triene, and tetraene conjugated fatty acid compounds as minor and trace constituents. Such materials almost invariably contain other absorbing bodies, consisting probably of oxidation and polymerization products, pigments, and other unknown compounds.

Ultraviolet absorption curves for typical samples of perilla oil, tallow, and a partially hydrogenated tallow before isomerization are shown in Fig. 1, and after isomerization in Fig. 2. Overlapping of absorption by the various fatty acid constituents, and the presence in some cases of absorption by extraneous compounds are apparent. For the partially hydrogenated tallow 14, in both Fig. 1 and Fig. 2, the absence of absorption maxima or inflections in the regions of absorption by conjugated triene acids (near 268 and 278 mμ) and by conjugated tetraene acids (near 301 and 316 mμ) indicates with a high degree of sensitivity absence of triene and

[4] L. J. van der Hulst, Rec. Trav. Chim. des Pays-Bas 54, 639, 644 (1935).
[5] A. Dingwall and J. C. Thomson, J. Am. Chem. Soc. 56, 899 (1934).
[6] H. P. Kaufmann, J. Baltes, and S. Funke, Fette u. Seifen 45, 302 (1938).
[7] W. R. Brode, J. W. Patterson, J. B. Brown, and J. Frankel, Ind. Eng. Chem. Anal. Ed. 16, 77 (1944).
[8] J. P. Kass, *Protective and Decorative Coatings*, edited by J. J. Matiello (John Wiley and Sons, New York, 1944), Chap. 12.

tetraene fatty acid constituents, although appreciable absorption is observed in these regions. A weak, broad, smooth absorption band near 280 mμ has been observed with many selectively hydrogenated tallows and their soaps. A similar broad, smooth absorption band, but with a maximum near 264 mμ, has been observed in alkali-isomerized samples of partially hydrogenated lards and tallows and their soaps, and methyl oleate. While the origin of these bands near 280 and 264 mμ is at present obscure, they cannot be attributed to conjugated triene fatty acid components.

Ultraviolet absorption curves are shown in Fig. 1 for purified β-eleostearic acid, 10,12 linoleic acid, methyl oleate, and methyl stearate. Data for these and other purified fatty acids are summarized in Table I.

The specific extinction coefficient 119 at 231 mμ obtained for the 10,12 linoleic acid sample (prepared from dehydrated castor oil) in neohexane is in satisfactory agreement with published values[9] for 9,11 and 10,12 linoleic acid. The shape of the curve in the region 260–280 mμ indicates the presence of a small impurity of conjugated triene acid, probably less than 0.1 percent. A wave-length of 232 mμ was adopted for analytical purposes in order to coordinate data with analyses involving alkali isomerization.

The specific extinction coefficient 214 at 267 mμ obtained for the β-eleostearic acid (prepared from tung oil) in neohexane is in satisfactory agreement with published values,[10] and was adopted as a standard for use in analysis for conjugated triene acids. The weak maximum near 313 mμ in Fig. 1 for this sample indicates the presence of a small impurity of conjugated tetraene acid. The higher value shown for neohexane solutions as compared with alcohol applies only to freshly prepared solutions.[11] A wave-length of 268 mμ was adopted for analytical purposes, since maxima in solutions of samples are usually at 268 mμ.

The only data available to use as standards in the analysis for tetraene conjugated acids are those for parinaric acid,[6] a naturally occurring C_{18} acid, and those for a C_{20} conjugated tetraene fatty acid isolated from isomerized arachidonic acid.[12] For the former acid, absorption maxima at 292, 307, and 320 mμ were reported, with a k value of about 203 at the 320 mμ maximum. For the latter acid, maxima were reported at 286, 300, and 315 mμ, with a k value of 220 at 315 mμ. Since conjugated tetraene acids are usually present only in trace amounts in vegetable oils and animal fats, it matters little which of these acids is chosen as a standard. However, until further work is done on such acids, we have preferred to use the data on the conjugated tetraene acid from isomerized arachidonic acid as a standard for analysis, since we have observed an absorption maximum near 316 mμ and an inflection near 301 mμ in many fatty materials.

Examination of the absorption curves of Fig. 1 and a large number of curves for vegetable oils and animal fats leads to the following conclusions: (*a*) reliable analyses for conjugated diene components will in many cases necessitate correcting the observed absorption at 232 mμ for absorption by the carboxyl radical (see Fig. 1, curve for methyl stearate 5 and methyl oleate 4) and for multiple C=C groups (see steep rise in curve below 220 mμ for perilla oil 18, which is rich in linolenic acid; also note in Table I that the single C=C group apparently has no appreciable absorption at 232 mμ); (*b*) reliable analyses for conjugated triene components will involve correction of observed absorption near 268 mμ for background absorption by conjugated diene and unknown constituents; (*c*) reliable analyses for conjugated tetraene components will involve correction of observed absorption near 316 mμ for background absorption by conjugated triene and unknown constituents. The same considerations apply also to the examination of curves such as

[9] Kass (reference 8) gives 115 at 232 mμ for both 9,11 and 10,12 linoleic acids; Kerns, Belkengren, Clark, and Miller, J. Opt. Soc. Am. 31, 271 (1941) report 116 at 232 mμ for 10,12 linoleic acid; our measurements with a comparator on van der Hulst's curves (reference 4, Fig. 3, page 641 and Fig. 6, page 649) indicate values of 119 and 126 for 9,11 linoleic acid in hexane.

[10] Kass (reference 8) reports a value of 215 at 268 mμ for both β- and pseudo-eleostearic acids; Dingwall and Thomson (reference 5) about 189 for β-eleostearic acid in ethanol; our measurements with a comparator on van der Hulst's curve (reference 4, Fig. 5, page 642) indicates about 194 for β-eleostearic acid in hexane.

[11] Instability of preparations of α-eleostearic acid have been discussed by R. T. O'Connor, D. C. Heinzelman, A. F. Freeman, and F. C. Pack, Ind. Eng. Chem. Anal. Ed. (in press).

[12] D. T. Mowry, W. R. Brode, and J. B. Brown, J. Biol. Chem. 142, 671 (1942).

those of Fig. 2 in the analysis for the non-conjugated fatty acids by the method of alkali-isomerization.

In the equations which follow, subscripts 2, 3, and 4 refer to the number of C=C bonds, and subscripts 232, 268, etc., refer to wave-lengths. The specific extinction coefficient due to conjugated diene fatty acid components will be approximately

$$k_2 = k_{232} - 0.07 - \cdots \text{ for fats and esters,} \quad (1)$$

$$k_2 = k_{232} - 0.03 - \cdots \text{ for soaps and acids,} \quad (1a)$$

where k_{232} is the observed coefficient at 232 mμ; 0.07 and 0.03 are the specific extinction coefficients at 232 mμ for purified methyl stearate and stearic acid, respectively (see Fig. 1 and Table I). Evidence that the value 0.07 is applicable to glycerides as well as to monohydric esters exists in the observation that many hydrogenated tallows having only traces of polyunsaturated constituents show k values of about 0.09 at 232 mμ. The equations as written are applicable principally to materials having small proportions of non-conjugated polyunsaturated constituents. For materials containing substantial proportions of linoleic and linolenic acids, appropriate terms will have to be added to these equations as reliable data become available.

An approximate correction for overlapping and background absorption in the regions of absorption by the conjugated triene and tetraene fatty acid compounds may be accomplished by determining the prominence of the absorption maxima near 268 and 316 mμ in terms of specific extinction coefficient differences at and near the maxima.

It can be shown that for a solution of a mixture containing β-eleostearic acid and one or more absorbing constituents having linear absorption curves between 262 and 274 mμ, the specific extinction coefficient due to the β-eleostearic acid in the mixture is

$$k_3 = 2.8[k_{268} - \tfrac{1}{2}(k_{262} + k_{274})], \quad (2)$$

where k_{268}, k_{262}, and k_{274} are observed coefficients for the mixture at the indicated wave-lengths, and 2.8 is a constant evaluated from measurements on solutions of pure β-eleostearic acid.

The derivation and application of this relation is illustrated in Fig. 3. For the pure acid in alcohol solution, the ratio $k_{268}/[k_{268} - \frac{1}{2}(k_{262} + k_{274})] = k/\Delta k = 209/75.3 = 2.8$. In a solution of β-eleostearic acid of unknown concentration, the value of Δk will be proportional to the concentration and the constant 2.8 will be independent of the concentration. In a mixture containing the acid and absorbing constituents having linear absorption between 262 and 274 mμ, the contribution of the latter to Δk will be zero, and thus the concentration of β-eleostearic acid in the mixture is still proportional to Δk. Hence the expression for k_3 in Eq. (2).

Applying Eq. (2) to curve B in Fig. 3 for a typical tallow, for which the observed values of k_{268}, k_{262}, and k_{274} are 0.0542, 0.0508, and 0.0434, respectively, we calculate $\Delta k = 0.0071$ and $k_3 = 0.0200$, the coefficient due to eleostearic acid. This leaves a value of 0.0342 due presumably to extraneous constituents. Verification of this result is illustrated by curve C, which was obtained by subtracting from curve B increasing fractions of values of curve A until no maxima or minima remained in the resulting curve. The value of k at 268 mμ in curve C, representing tallow 14 minus 0.009 percent β-eleostearic acid, was 0.0353, which is in good agreement with the value 0.0342 calculated by use of Eq. (2) for the background absorption. Thus in this example, less than half the observed absorption at 268 mμ is due to conjugated triene fatty acid constituents.

It should be pointed out that the value of the constant 2.8 in Eq. (2) varies somewhat for the different isomers of eleostearic acid, for different solvents (Table I), and for different slit widths. However, for β- or pseudo-eleostearic acids, in neohexane or alcohol, and with spectrum band widths less than 2 mμ, any deviations in this value are within about 4 percent and thus give rise to much smaller errors than would be made by neglecting background correction.

Similar treatment in the region of the longest wave-length absorption band of conjugated tetraene acids leads to the following relation for the specific extinction coefficient of conjugated tetraene acids in the presence of other constituents having linear absorption curves between 310 and 322 mμ:

$$k_4 = 2.5[k_{316} - \tfrac{1}{2}(k_{310} + k_{322})], \quad (3)$$

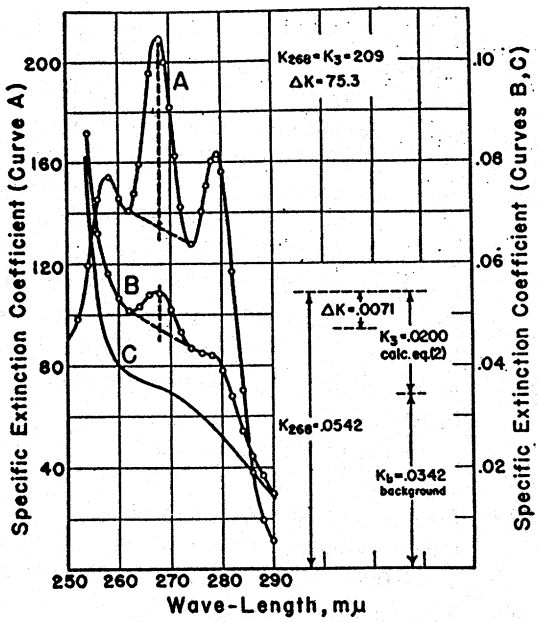

FIG. 3. Absorption curves: *A*, purified β-eleostearic acid 2 in ethanol; *B*, tallow 16 in neohexane; *C*, calculated curve of "background" absorption, i.e., tallow 16 minus 0.009 percent β-eleostearic acid.

where k_{316}, k_{310}, and k_{322} are the observed coefficients of the mixture, and 2.5 is a constant evaluated from isomerized arachidonic acid.[13] Background absorption in this region is in general more nearly linear than is the case in the conjugated triene region.

The concentrations of conjugated diene, triene, and tetraene constituents, expressed as percent of fatty acids, present in a given sample are calculated by the following equations:

$$C_2 = 100k_2/119, \quad (4)$$

$$C_3 = 100k_3/214, \quad (5)$$

$$C_4 = 100k_4/220, \quad (6)$$

where k_2, k_3, and k_4 are the corrected specific extinction coefficients calculated from Eqs. (1) or (1a)–(3), and the constants in the denominators are the coefficients for the standards in Table I.

Results on the analysis of typical samples for conjugated constituents appear in Tables V and VI in conjunction with other data.

[13] In the absence of detailed data on the conjugated tetraene acid isolated from isomerized arachidonic acid (reference 12), this constant was tentatively evaluated from data of Beadle and Kraybill (reference 2) for isomerized arachidonic acid. The authors are indebted to B. W. Beadle for the necessary data for this calculation.

NON-CONJUGATED CONSTITUENTS

The method of Mitchell, Kraybill, and Zscheile[1] for the analysis of oils and fats for linoleic and linolenic acids, and its extension by Beadle and Kraybill[2] to arachidonic acid, involves heating 0.1 gram of sample in 10 ml of 1.3*N* potassium hydroxide-ethylene glycol solution for 25 minutes at 180°C, cooling, diluting to 250 ml with 99 percent ethanol, settling, filtering, and measuring the ultraviolet absorption of the final solution against that of a reagent "blank" solution treated in the same way.

The principal difficulties encountered in attempting to apply this method to materials having very low proportions of non-conjugated polyunsaturated fatty acid constituents were: (*a*) the KOH-ethylene glycol isomerization medium develops a strong and variable ultraviolet absorption on heating in the presence of air; (*b*) the background absorption by extraneous compounds is relatively high (Fig. 2); and (*c*) the proportion of conjugated (particularly diene) to non-conjugated components is relatively high, raising the question as to what happens to the conjugated components during the isomerization treatment.

The first difficulty was usually manifested by poor reproducibility of analyses for linolenic and arachidonic acids. In some cases the observed spectral densities were actually negative, indicating that the density of the solute was less than the difference in density between two blanks run in the constant-temperature bath at the same time, thus making impossible the accurate analysis for small proportions of these constituents.

Improved transparency of the reagent solution was obtained by protecting the solutions during heating with a nitrogen atmosphere, but the reproducibility of absorption was not greatly improved in the first attempts. Further experiments along this line were abandoned when it was found that substitution of glycerol for ethylene glycol as a saponification and isomerization medium overcame previous difficulties without the added complication of excluding air.

The absorption and reproducibility of heated alkaline glycerol and ethylene glycol solutions are compared in Fig. 4 and Table II. The substantial gain in reproducibility and transparency by use

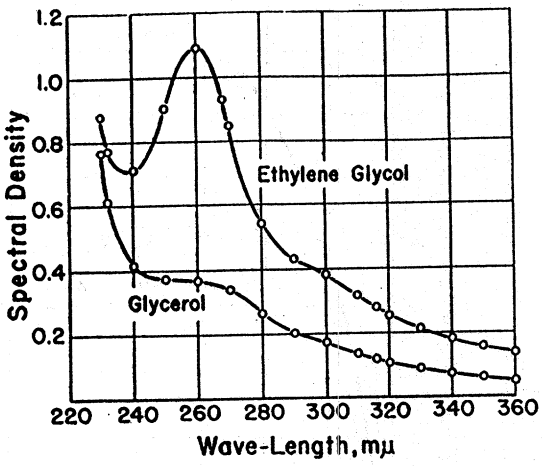

FIG. 4. Absorption curves for 1.30*N* KOH ethylene glycol and 11 percent KOH-glycerol solutions heated in the presence of air for 25 minutes at 180°C. Spectral densities are for 1-cm layers of 10 percent solutions in absolute ethanol, compared with a 1-cm layer of absolute ethanol.

of glycerol, together with application of corrections to isomerized samples for absorption by extraneous compounds, has made possible greatly improved sensitivity and accuracy in analysis for linolenic and arachidonic acids. The relatively high absorption below 230 mμ, caused by the potassium hydroxide in the solutions, is not troublesome since relatively high dilutions of the 10 percent solutions are required for absorption measurements on isomerized fatty materials in the diene region.

A modified analytical procedure applicable to a wide variety of oils, fats, soaps, and other fatty materials was devised. Standardization was accomplished by following spectrophotometrically the effect of time of heating on the isomerization reaction, using purified linoleic and linolenic acids.

The linoleic acid standard, prepared from corn oil by standard methods, had an iodine value of 181.9 and an acid value of 198.9. Spectrophotometric examination indicated the presence of approximately 0.3 percent linolenic acid, 1.7 percent conjugated diene, and 0.02 percent conjugated triene acids, and hence a purity not greater than 98.0 percent. The linolenic acid standard, prepared from perilla oil by standard methods, had an iodine number of 273.5 and an acid value of 200.6. Spectrophotometric examination indicated the presence of approximately 1.5 percent conjugated diene and 0.1 percent non-conjugated tetraene acids, and hence a purity not exceeding 98.4 percent. No purified arachidonic acid was available for the standardization tests in glycerol.

Curves showing relation of time of heating at 180°C to specific extinction coefficients (in absolute ethanol) at the absorption maxima of the conjugated products formed are shown in Fig. 5 for 0.1-gram portions of the pure acids each in 11.0 grams of glycerol solution containing 11.0 percent KOH by weight. Although a heating time of about 45 minutes would appear to be optimum for analytical purposes, a slightly less favorable time of 30 minutes was actually selected in order to use the data of Beadle and Kraybill[2] for arachidonic acid. The observed coefficients, their values adjusted to 100 percent purity of the acids, and for comparison the coefficients reported by Beadle and Kraybill,[2] are shown in Table III. Complete absorption curves for the treated acids are shown in Fig. 2.

In order to determine the effect of the alkali treatment on conjugated fatty constituents, measurements were made under these same conditions on conjugated materials, with the results shown in Fig. 6 and Table IV. It is apparent that the conjugated acids and glycerides are little affected by this treatment. Hence in the analysis of a sample for non-conjugated constituents by the alkali-isomerization method, correction must

TABLE II. Absorption and reproducibility for 1.30*N* KOH-ethylene glycol and 11 percent KOH-glycerol solutions heated in the presence of air for 25 minutes at 180°C. Spectral densities for 10 percent solutions in absolute ethanol, 1 cm at 260 mμ, 5 cm at 316 mμ (i.e., thicknesses and concentrations commonly used for analysis of animal fats) *vs.* equal layers of absolute ethanol. Each group of six samples was run simultaneously in the constant temperature bath.

	Spectral density			
	KOH-ethylene glycol		KOH-glycerol	
	260 mμ	316 mμ	260 mμ	316 mμ
	1.13	2.79	.359	.585
	1.18	2.91	.362	.590
	1.02	2.69	.377	.600
	1.09	2.71	.377	.605
	1.22	2.88	.377	.605
	0.96	2.51	.370	.595
Mean	1.10	2.75	.370	.597
Standard deviation	±0.10	±0.15	±.008	±.008

be made for conjugated constituents originally present in appreciable amounts.

In addition, correction of ultraviolet absorption data on the isomerized product must be made for background absorption by extraneous compounds (see Fig. 2 for absorption curves of typical alkali-isomerized fatty materials). This background absorption may be corrected for in the same way as proposed above in the analysis for conjugated constituents.

Thus after the isomerization treatment, absorption near 232 mμ will be contributed by conjugated diene components originally present in the fatty material; by conjugated diene components from isomerized linoleic, linolenic, and arachidonic acid compounds; and by extraneous compounds. Absorption near 268 mμ will be contributed by conjugated triene components originally present; by conjugated triene components from isomerized linolenic and arachidonic acid constituents; and by extraneous compounds. Absorption near 316 mμ will be contributed by conjugated tetraene components originally present; by conjugated tetraene components from isomerized arachidonic acid compounds; and by extraneous compounds.

In the equations which follow, all primed coefficients refer to the isomerized product in absolute ethanol, and unprimed coefficients refer to the untreated material dealt with in Eqs. (1)–(6); the subscripts have the same meaning as before.

The coefficient at 232 mμ corrected for conjugated diene material originally present is,

TABLE III. Specific extinction coefficients for alkali-isomerized pure polyunsaturated fatty acids.

Sample No.	Acid	Wavelength, mμ	Specific extinction coefficients [a]	[b]	[c]
7	Linoleic	232	89.1	88.9	86.0[d]
8	Linolenic	232	60.8	60.0	60.9[d]
8	Linolenic	268	52.6	53.4	53.2
—	Arachidonic	232	—	59.3	59.3[d]
—	Arachidonic	268	—	53.4	53.4
—	Arachidonic	316	—	22.6	22.6

[a] Observed values, in glycerol 30 minutes at 180°C.
[b] Values used for analysis, in glycerol 30 minutes at 180°C: observed values adjusted to 100 percent purity of acid; arachidonic acid coefficients assumed valid for these conditions.
[c] Values reported by Beadle and Kraybill (see reference 2), in ethylene glycol 25 minutes at 180°C.
[d] At 234 mμ.

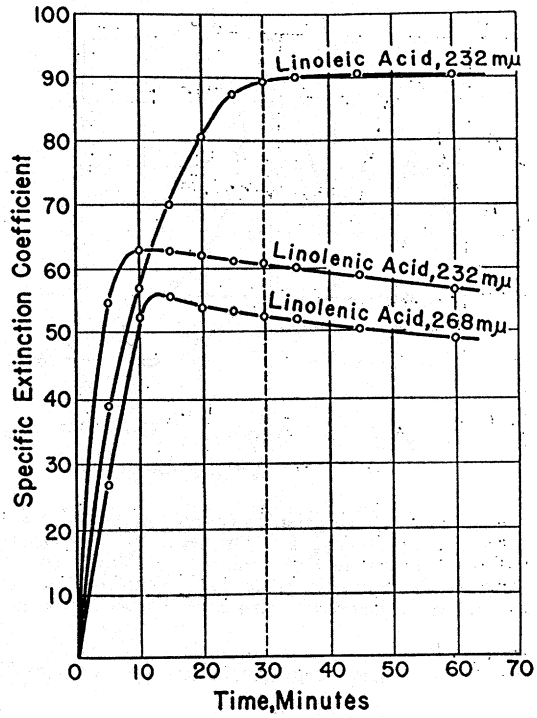

FIG. 5. Specific extinction coefficients in absolute ethanol for 0.1-gram samples of purified linoleic and linolenic acids heated for various lengths of time at 180°C in 11 percent KOH-glycerol solution.

approximately:

$$k'_2 = k'_{232} - k_{232} + 0.04 \text{ for fats and esters,} \quad (7)$$

$$k'_2 = k'_{232} - k_{232} \text{ for soaps and acids,} \quad (7a)$$

where k'_{232} and k_{232} are the observed specific extinction coefficients of the material after and before isomerization. The constant 0.04 compensates for the fact that fats and esters are converted to soaps by the hot alkali treatment (see Eqs. (1) and (1a)). This term is important only for samples containing less than about 1 percent of linoleic acid.

The coefficient at 268 mμ corrected for background absorption is approximately:

$$k'_3 = 4.1[k'_{268} - \tfrac{1}{2}(k'_{262} + k'_{274})], \quad (8)$$

where k'_{262}, k'_{268}, and k'_{274} are the observed specific extinction coefficients, and the constant 4.1 was evaluated from measurements on ethanol solutions of the isomerized pure linolenic acid preparation 8. No explanation is advanced at this time for the fact that the value of this constant is

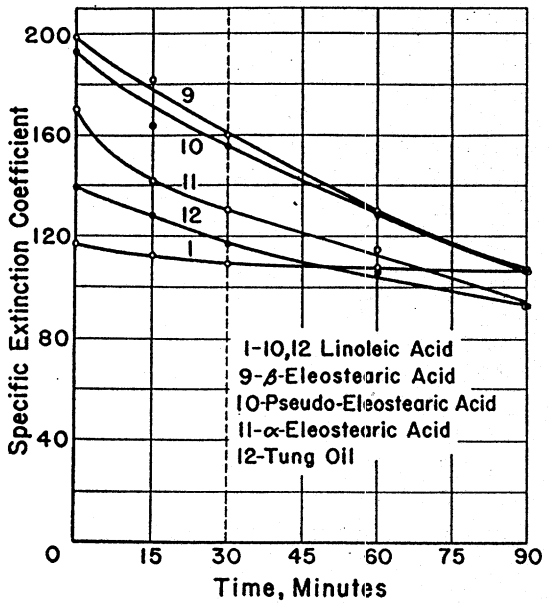

FIG. 6. Specific extinction coefficients in absolute ethanol for conjugated fatty acids and tung oil before and after heating for various lengths of time at 180°C in KOH-glycerol solution.

considerably higher than was obtained for β-eleostearic acid used in Eq. (2).

The coefficient at 268 mμ corrected for conjugated triene material originally present is approximately:

$$k''_3 = k'_3 - k_3, \qquad (9)$$

where k'_3 is given by Eq. (8) and k_3 by Eq. (2). This correction is usually small, and a coefficient of unity for k_3 may be used instead of the value 0.8 indicated by Table IV to compensate for the application in Eq. (8) of the factor 4.1 to the undestroyed conjugated material as well as to that formed by isomerization.

The coefficient at 316 mμ corrected for background absorption is approximately:

$$k'_4 = 2.5[k'_{316} - \tfrac{1}{2}(k'_{310} + k'_{322})], \qquad (10)$$

where k'_{310}, k'_{316}, and k'_{322} are the observed specific extinction coefficients and the constant 2.5 was evaluated from isomerized arachidonic acid.[2,18] The final corrected coefficient at 316 mμ, is assumed to be:

$$k''_4 = k'_4 - k_4, \qquad (11)$$

where k'_4 is given by Eq. (10) and k_4 by Eq. (3).

Since the specific extinction coefficient of a mixture at a selected wave-length is equal to the sum of the specific extinction coefficients of the components, each multiplied by its proportion in the mixture, simultaneous equations may be set up for the system and solved for the proportion of each component. Using the standardized data of Table III and the corrected specific extinction coefficients defined by Eqs. (7) or (7a), (9), and (11), these final equations for the KOH-glycerol procedure, with 30 minutes heating at 180°C, are:

$$x = 1.125k'_2 - 1.27k''_3 + 0.04k''_4, \qquad (12)$$

$$y = 1.87k''_3 - 4.43k''_4, \qquad (13)$$

$$z = 4.43k''_4, \qquad (14)$$

where x, y, and z are the concentrations, expressed as percent fatty acid, of linoleic, linolenic, and arachidonic acid constituents present in a given sample. If corrections are omitted for absorption by undestroyed conjugated and other extraneous constituents, the coefficients k'_2, k''_3, and k''_4 become the observed coefficients k'_{232}, k'_{268}, and k'_{316}, and the calculations indicated by the final equations become equivalent to those described by Mitchell *et al.*,[1] and Beadle and Kraybill.[2]

PROCEDURE FOR ANALYSIS

A sample of oil, melted fat, acid, ester, or powdered soap about 200 mg in size is weighed to the nearest milligram, dissolved in about 75 ml of neohexane[14] or in 95 percent ethyl alcohol,[15]

TABLE IV. Specific extinction coefficients in ethanol, and wave-lengths of absorption maxima for conjugated fatty materials before (k) and after (k') heating for 30 minutes at 180°C in KOH-glycerol solution. The ratio k'/k gives approximately the proportion of conjugated material undestroyed by the treatment.

Sample No.	Material	Before		After		
		λ	k	λ'	k'	k'/k
1	10,12 linoleic acid	231	117	231	110	.94
9	β-eleostearic acid	268	200	268	161	.81
10	Pseudo-eleostearic acid	267.5	193	267.5	156	.81
11	α-eleostearic acid	270	171	269	131	.77
12	Tung oil	270	140	269	118	.84

[14] Neohexane, Phillips Petroleum Company, technical grade. Isooctane (2,2,4-trimethylpentane), Rohm and Haas, Bureau of Standards certified grade, is also a satisfactory solvent. The transparency of these solvents was improved greatly by passage through a column of silica gel; see M. M. Graff, R. T. O'Connor, and E. L. Skau, Ind.

TABLE V. Analysis of tallow 15 for polyunsaturated fatty acids by different methods. [(a), (b), (c) were isomerized in KOH-glycerol; (d) was isomerized in KOH-ethylene glycol (no corrections made).]

Acid	(a) Proposed method, percent	(b) Correction for conjugation neglected, percent	(c) Corrections for background and conjugation neglected, percent	(d) Method of Mitchell *et al*[a] Beadle *et al*,[b] percent	(e) Method of Bradley and Richardson,[c] percent	(f) Method of Brode *et al*,[d] percent	(g) Method of Kass,[e] percent
Linoleic	3.39 3.39	4.55 4.53	4.25 4.23	4.35 4.15	—	—	—
Linolenic	.61 .64	.63 .66	.74 .75	.51 .57	—	—	—
Arachidonic	.68 .64	.70 .67	1.04 1.02	1.20 1.14	—	—	—
Conjugated diene	.86	—	—	—	.90	.90	.94
Conjugated triene	.011	—	—	—	.12	.14	.094
Conjugated tetraene	.002	—	—	—	.030	.030	.040

[a] See reference 1.
[b] See reference 2.
[c] See reference 3.
[d] See reference 7.
[e] See reference 8.

transferred quantitatively to a 100-ml volumetric flask, and made up to volume. Spectral densities are then measured on a Beckman spectrophotometer preferably at 2-mμ intervals from 324 to 306 mμ, 282 to 260 mμ, and 240 to 226 mμ, dilutions and cell lengths (1 to 5 cm) being adjusted so that, whenever possible, observed densities are between 0.2 and 0.8. Curves are plotted for examination, the necessary specific extinction coefficients are calculated, and the percentages of conjugated diene, triene, and tetraene fatty acid components present are computed by Eqs. (1) through (6).

Two additional samples of about 100 mg are weighed to the nearest half milligram in Pyrex-glass cups of approximately 1-ml capacity; these samples are for analysis in duplicate for nonconjugated constituents. The isomerization reagent is prepared by adding 17.5 grams of KOH (A.C.S. standard) to 100 ml of glycerol (U.S.P. XII) and heating to 200°C with constant stirring to dissolve the alkali and drive off excess water. The resulting reagent should have a KOH concentration of 10.9 to 11.0 percent by weight, and may be checked by titration. Three 6-×1-inch Pyrex-glass test tubes, serving as reaction tubes, are filled with 11.0-gram portions of the solution and then suspended to a depth of 4½ inches in a constant temperature bath operated at 180°C±0.5°.

When the temperature of the solution in a tube has reached 180°, the glass vessel containing a weighed sample is dropped into the tube, the tube is removed from the bath, swirled vigorously for one minute, then returned to the bath and covered with a crucible cover. At the end of a minute's heating in the bath, the tube is removed and inspected.[16] If the solution is clear, the tube is returned to the bath. If saponification or solution is not complete, the tube is again swirled a few times, the cover is replaced, and the tube returned to the bath. The procedure is repeated with the duplicate sample. An empty sample container is dropped in the third reaction tube, which serves as a reference blank.

Eng. Chem. Anal. Ed. 16, 556 (1944); also Nat. Bur. Stand. Tech. News Bull. No. 325, p. 37 (May 1944).

[15] Synthetic absolute methyl alcohol is also a suitable solvent. We are indebted to E. L. Borg of the United States Rubber Company for suggesting its use.

[16] It is essential that all samples saponify or dissolve within one to two minutes after being dropped into the reaction tube. Animal fats and fatty acids saponify within a minute. Monohydric esters saponify less readily. This may be remedied by adding 0.1 percent of pure palmitic or stearic acids to the KOH-glycerol reagent after the preliminary heating at 200°. In the case of soap samples, prompt solution is accomplished by wetting the sample with 3 drops of water or glycerol, stirring it to a homogeneous mass, and dropping sample and glass stirring rod into the reaction tube.

TABLE VI. Analysis of miscellaneous fatty materials for polyunsaturated constituents. Effect of variations in isomerization medium, size of sample, and temperature; reproducibility of results; and analysis of known mixtures.

Sample	No.	Iodine value	Non-conjugated acids, percent			Conjugated acids, percent			Total polyunsaturated acids, percent	
			Linoleic	Linolenic	Arachidonic	Diene	Triene	Tetraene	Spectrophotometric	Chemical
Tallow	13	57.5	4.8	0.46	0.29	0.68	0.008	0.001	6.24	8.3[a]
Tallow, hydrogenated	14	49.4	0.06	0.00	0.00	0.15	0.000	0.000	0.21	—
Oleic acid, purified	20	91.1	1.05	0.04	0.00	0.52	0.000	0.000	1.61	—
Perilla oil	18	207.3								79.9[b]
isomerized in glycerol			13.1	55.6	1.3	2.2	0.07	0.02	72.3	
isomerized in ethylene glycol			13.1	55.1	1.1	—	—	—	71.6	
Soybean oil, coldpressed	19	—								—
mean of 20 runs			43.25	4.12	0.98	2.6	0.01	0.000	51.0	
standard deviation			±0.21	±0.09	±0.02					
Tallow	17	56.7								8.8[a]
mean of 20 runs			4.66	0.52	0.23	0.90	0.02	0.000	6.33	
standard deviation			±0.09	±0.03	±0.03					
Tallow	21	—								—
0.1 g[c]			4.1	0.48	0.19	—	—	—		
0.2 g			3.8	0.46	0.18	—	—	—		
0.3 g			3.0	0.44	0.18	—	—	—		
Linoleic acid concentrate	22	168.0								—
175°C			62.2	6.8	0.26	—	—	—		
180°[c]			64.5	7.1	0.25					
185°			66.5	7.2	0.21					
Mixture	23	—								—
Calculated			9.1	0.98	0.00	0.36	0.001	0.000	10.44	
Found			9.1	0.90	0.00	0.32	0.001	0.000	10.32	
Mixture	24	—								—
Calculated			4.6	0.50	0.00	0.18	0.0004	0.000	5.28	
Found			4.9	0.40	0.00	0.18	0.0005	0.000	5.48	
Mixture	25	—								—
Calculated			0.48	0.05	0.00	0.02	0.000	0.000	0.55	
Found			0.58	0.05	0.00	0.02	0.000	0.000	0.65	

[a] Thiocyanometric method, assuming the presence of saturated, oleic, and linoleic acids only.
[b] Thiocyanometric method: linoleic acid, 14.7 percent; linolenic acid, 65.2 percent.
[c] Standard.

Thirty minutes after the sample is dropped into the reagent tube, the tube is removed and cooled under tap water, 20 ml of absolute ethanol[15] is added, and the mixture is stirred. The tube is placed in a beaker of hot water over low heat, and stirring is continued until the viscous product dissolves completely. The solution is then transferred quantitatively to a 100-ml volumetric flask and made up to volume with absolute ethanol.[15] No settling and filtering are required when absolute alcohol is used as a solvent. As little as 1 percent water in the solvent causes a precipitate.

Spectral density measurements are made exactly as specified in the analysis for conjugated constituents, except that the "blank" is the heated reagent and must be diluted to the same extent as the sample solutions. The required specific extinction coefficients are calculated, and the percentages of linoleic, linolenic, and arachidonic acids in the sample are computed by means of Eqs. (7) through (14).

If the calculated value for k'_3 in Eq. (8) is zero or negative, no linolenic acid is present. A further criterion is used when small positive values for k'_3 occur: if the plotted curve of density *vs.* wavelength shows no evidence of simultaneous absorption maxima or inflections near wave-lengths 268 and 278 mμ, linolenic acid is absent.

The complete analysis of a tallow sample for

polyunsaturated fatty acid constituents is shown in Table V. The data show (*a*) results by the proposed method; (*b*) the effect on the results of omitting correction for undestroyed conjugated constituents; (*c*) the effect of neglecting in addition corrections for background absorption; (*d*) results obtained for the non-conjugated acids by previous methods;[1,2] and (*a*), (*e*), (*f*), (*g*) comparison of different methods for the analysis of conjugated constituents.

The data illustrate that, in fats of this type (1) the correction is appreciable for the presence of undestroyed conjugated diene acids, but negligible for conjugated triene and tetraene acids; (2) large errors may be made in the results if background absorption is neglected; and (3) the methods and standardization data of other investigators for conjugated acid assay give essentially the same results but are probably greatly in error, at least for triene and tetraene acids. The dependent nature of the simultaneous analysis for linoleic, linolenic, and arachidonic acids should be kept in mind; for example, any large error in the determination of arachidonic acid (the only independently determined non-conjugated acid) will produce an appreciable error in the values calculated for linolenic and linoleic acids.

Results on the spectrophotometric analysis of some typical fatty materials for polyunsaturated constituents are shown in Table VI. Iodine values and percent linoleic acid obtained by the thiocyanometric method are shown where data were available for the chemical method. The spectrophotometric analyses are reported as percent acid in total sample, whether the sample be a fat, ester, or acid. The proportions of oleic and saturated acids can be calculated from the proportions of polyunsaturated constituents indicated (corrected to the basis of percent acid in total fatty acids), their theoretical iodine numbers, and the iodine number of the sample.

Mild selective hydrogenation (sample 14) reduces the linoleic and linolenic acid content of tallow to extremely low levels, and completely removes tetraene and triene conjugated constituents. The data for purified oleic acid (sample 20) illustrate the value of the method in detecting low amounts of polyunsaturated impurities in fatty materials used in research studies, for example, on oxidation. Neglect of background absorption in cases such as 14 and 20 would lead to completely erroneous results.

Application of the method to vegetable oils having substantial proportions of linoleic and linolenic acid components is also shown (samples 18 and 19). The principal value of the modified method in such cases is to furnish more reliable data for the less abundant constituents. The presence of tetraenoic fatty acid constituents in these oils is of interest.

In the case of the perilla oil sample, results of analyses in which both glycerol and ethylene glycol were used as isomerization solvents are in good agreement. The value for arachidonic acid obtained by the glycerol procedure is more reliab e for reasons previously discussed.

Data in Table VI (sample 21) indicate that the size of the sample used in the analysis for non-conjugated constituents is rather critical, and should not differ from the standardized 0.1-gram size by more than about 10 percent. Similar deviations have been obtained with samples of soybean oil and partially hydrogenated tallow, indicating the need for further study of the isomerization reaction.

Tests on a tallow and a linoleic acid concentrate (sample 22) indicate that the temperature at which the isomerization reaction is carried out should be maintained constant to about ±0.5°C.

The high reproducibility of the method is illustrated by the standard deviation of 20 analyses (runs in duplicate on ten different days) for samples of soybean oil and tallow. All other results shown in Table VI are the average of duplicate analyses. No difficulty has been experienced in attaining this reproducibility when the procedure specified is closely followed and when saponification or solution occurs within one or two minutes after the sample is dropped into the reaction tube.

The accuracy of the method was tested by analyzing purified methyl esters of linoleic, linolenic, and oleic acids, and their mixtures. The calculated and found values of the diene and triene constituents are shown in Table VI for three mixtures which roughly simulate the composition of lard, tallow, and selectively hydrogenated tallow. The agreement between calculated and observed values is good.

The accuracy of the method is probably not so high as the reproducibility. Factors contributing to the uncertainty of results include imperfections in correction for interfering constituents, incomplete identification of impurities in the standards used, and the possible presence in some samples of isomers having conjugation rates different from those of the standards. Agreement with the chemical method is best when linoleic acid is present in much greater abundance than acids of higher unsaturation (e.g., lard and tobacco seed oil). It is conservatively estimated that the errors of the method are within ±10 percent of the quantity present when that quantity is near 10 percent, ±25 percent near 1 percent, and that the results are at least correct in order of magnitude when the quantity present is 0.1 percent or less.

The application of the method to a wider variety of fatty materials will be published elsewhere.[17]

SUMMARY

A method is described for the simultaneous spectrophotometric determination of non-conjugated and conjugated diene, triene, and tetraene fatty acid constituents in vegetable oils, animal fats, their soaps, and purified fatty acid preparations. The method involves measurement of the ultraviolet absorption of a sample before and after isomerization. Correction of the data for absorption by extraneous compounds and use of alkaline glycerol as an isomerization medium result in greater sensitivity and accuracy in the analysis for minor and trace proportions of these polyunsaturated constituents.

[17] B. A. Brice, M. L. Swain, B. B. Schaeffer, and W. C. Ault, *Oil and Soap* (in press).